

Research article

Variation in the Integration Site for Capsule Gene Cluster in the Genome among Strains of *Lactococcus garvieae*

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ABSTRACT—*Lactococcus garvieae* is known as a pathogen of freshwater and marine fish species worldwide. *L. garvieae* isolates of serotype I have been divided into two serological phenotypes, namely KG⁻ and KG⁺, which are differentiated by the presence or absence of polysaccharide capsule, and a phenotypic change from KG⁻ to KG⁺ occurs during repeated subculturing. When we subcultured laboratory collections of *L. garvieae* KG⁻ strains repeatedly, they were divided into two groups. One group consisted of the strains which changed phenotypically after a relatively small number of times of subculturing, and the other group consisted of those which hardly changed. Genetic analyses revealed that the capsule gene cluster of the strains in the former group was integrated in a plasmid (CPS plasmid) and that in the latter group it was integrated in the chromosome or in both CPS plasmid and chromosome simultaneously. In the present study, we found three novel CPS plasmids. These plasmids were similar in structure to the CPS plasmid pBSLG13015 which was previously found in *L. garvieae* filefish isolates. In addition, we found a novel integration site of chromosomal capsule gene cluster. The majority of strains isolated before 1991 were those whose capsule gene cluster was integrated in the CPS plasmids.

Key words: *Lactococcus garvieae*, capsule gene cluster, CPS plasmid, KG⁻, KG⁺, phenotypic change

Lactococcus garvieae is known as a fish pathogen causing damages in aquaculture industry worldwide. In Japan lactococcosis occurs frequently among *Seriola* species, but other marine fish species including Japanese flounder *Paralichthys olivaceus*, Pacific bluefin tuna *Thunnus orientalis* and filefish, thread-sail filefish *Stephanolepis cirrhifer* and black scraper *Thamnaconus modestus*, are also susceptible to the disease (Kawanishi *et al.*, 2006; Minami *et al.*, 2012; Yoshida, 2016). *L. garvieae* isolates of serotype I have been divided into two serological phenotypes, namely KG⁻ and KG⁺ types, which are differentiated by an agglutination test using KG⁻ and KG⁺ antisera (Kitao, 1982). The KG⁻ type produces a capsule on its cell-surface, is pathogenic to fish and possesses a gene cluster for capsular polysaccharide biosynthesis (capsule gene cluster) in the chromosome (Yoshida *et al.*, 1997; Miyauchi *et al.*, 2012). It was reported that the change from KG⁻ to KG⁺ type occurred during repeated subculturing (Kitao, 1982), and it is speculated that the capsule gene cluster of KG⁻ type

is lost during subculturing (Miyauchi *et al.*, 2012).

Recently we found that the phenotypic change from KG⁻ to KG⁺ occurred more easily in *L. garvieae* isolates derived from filefish than those from other fish species, and reported that all the tested filefish KG⁻ strains possessed a plasmid carrying the capsule gene cluster (CPS plasmid), which we named pBSLG13015 (Kanai *et al.*, 2017). This plasmid was not detected in KG⁺ derivatives of filefish KG⁻ strains. Therefore, it was considered that the phenotypic change in filefish isolates was attributed to the loss of the CPS plasmid.

Approximately two decades ago we noticed that there were two groups in stocked *L. garvieae* KG⁻ strains derived from yellowtail *Seriola quinqueradiata*. One group consisted of the strains in which the phenotypic change occurred after a relatively small number of times of subculturing, and the other group consisted of those in which the phenotypic change hardly occurred (Fig. 1). At that time, we could not clarify the reason for the presence of these two groups, but, as mentioned above, the fact that *L. garvieae* strains from filefish possessed the CPS plasmid promoted us to investigate the presence or absence of CPS plasmids in laboratory-stocked

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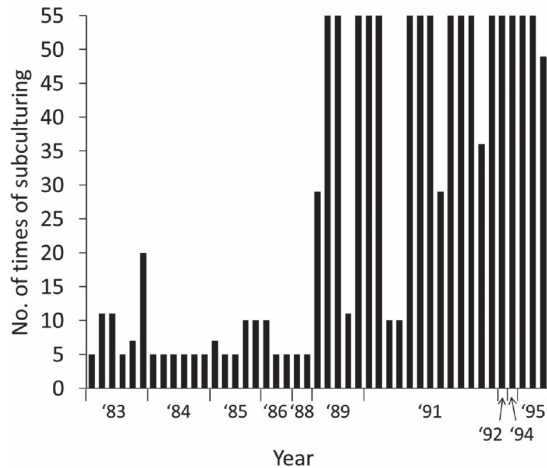


Fig. 1. Phenotypic change occurred in *L. garvieae* KG⁻ strains isolated between 1983 and 1995 by repeated subculturing on Todd Hewitt agar. Each bar represents the number of times of subculturing that caused a change to KG⁺ phenotype in each strain. The bars reached 55 mean that those strains did not change to KG⁺ by 55 times of subculturing.

L. garvieae serotype I strains derived from diseased marine fish species other than filefish. In addition, at that time we also noticed that antibiotic susceptibility was different between the two groups, that is, strains of the former group were susceptible to erythromycin, but more than half of those of the latter group were resistant to this antibiotic. Thus, in the present study relationship between the integration site of the capsule gene cluster and antibiotic susceptibility of the stocked *L. garvieae* strains was also investigated.

Materials and Methods

Bacterial strains

A total of 152 *L. garvieae* strains isolated from diseased marine fish at aquaculture sites in western Japan were used in this study (Table 1). From these strains, NUF18, NUF462, Ns12sousui23 and Ns15sousui29 were chosen for genetic analysis as representative strains (Table 2). The serological phenotype of the 152 strains was determined by a slide agglutination test using KG⁻ and KG⁺ rabbit antisera (Kanai *et al.*, 2017). Before the test, each strain was divided by colony morphology (see Results). The strains were cultured on Todd Hewitt (TH) agar (Difco) at 27°C and stocked by freezing at -80°C in a deep freezer or at -196°C in liquid nitrogen.

Detection of capsule gene cluster

The presence or absence of *L. garvieae* capsule gene cluster in the KG⁻ and/or KG⁺ phenotype of each strain was examined by the PCR method described previously (Kanai *et al.*, 2017) with some modifications. Primer pairs used were Lg-Cps-38F/Lg-Cps-36R for the

Table 1. Source of *Lactococcus garvieae* strains used in this study

Year	No. of strains						Total	
	<i>Seriola</i>			<i>Paralichthys olivaceus</i>	<i>Thunnus orientalis</i>	<i>Scomber japonicus</i>		<i>Girella punctata</i>
	<i>quinqueradiata</i>	<i>dumerilii</i>	<i>lalandi</i>					
1983	10						10	
1984	7						7	
1985	15						15	
1986	8						8	
1987	8						8	
1988	11						11	
1989	25						25	
1991	19						19	
1992	5						5	
1993				1			1	
1994	1						1	
1995	3						3	
2002				4			4	
2005		1					1	
2006	1						1	
2007	2					1	3	
2008	2		1				3	
2009	3	1					4	
2011	1	2	3			1	7	
2012		2		1	2		5	
2013			2		1		3	
2014					1		1	
2015	2		1				3	
2016	1		2		1		4	
Total	124	6	9	6	5	1	152	

chromosomal gene cluster designed from *L. garvieae* Lg2 complete genome sequence (GenBank accession no. NC_017490) and Lg-pF2/Lg-pR2 for the plasmid gene cluster (Table 3). PCR was performed on C1000 Thermal Cycler (Bio-Rad) using KOD FX Neo Polymerase (Toyobo). The PCR reaction mixture (50 µL) contained 25 µL of 2 × PCR buffer for KOD FX Neo (Toyobo), 0.4 mM of each dNTP, 0.3 µM of each primer, 1 µL of template DNA and 0.5 U of KOD FX Neo Polymerase. The PCR condition was as follows; initial denaturation at 94°C for 2 min and 30 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 30 s and extension at 68°C for 9 min. The PCR products were electrophoresed in 0.8% agarose gel with Quick Load 1 kb Extend DNA Ladder (New England BioLabs) and stained with ethidium bromide.

Nucleotide sequence analysis of integration site of capsule gene cluster

The genomic DNAs of *L. garvieae* NUF18, NUF462, Ns12sousui23 and Ns15sousui29 were isolated using Wizard Genomic DNA Purification kit (Promega)

Table 2. Representative *L. garvieae* strains used for genetic analysis

Strain	Source			Phenotype	CPS plasmid	Chromosomal capsule gene cluster
	Fish	Prefecture	Year			
NUF18	Yellowtail	Nagasaki	1983	KG ⁻	+	-
NUF462	Yellowtail	Kumamoto	1989	KG ⁻	+	+
Ns12sousui23	Pacific bluefin tuna	Nagasaki	2012	KG ⁻	+	-
Ns15sousui29	Kingfish	Nagasaki	2015	KG ⁻	+	-

Yellowtail, *Seriola quinqueradiata*; kingfish, *S. lalandi*; Pacific bluefin tuna, *Thunnus orientalis*.

Table 3. PCR primers used for the detection of capsule gene cluster in CPS plasmids and chromosomes of *L. garvieae*

Primer*	Sequence (5'-3')	Target
Lg-pF2	GTGCCATAAACATCGTCTGGTTG	CPS plasmids
Lg-pR2	ACCAACAGCGACAATAATGACG	CPS plasmids
Lg-Cps-1F	AGCAGCGTAGGCTATAGAAGCA	CPS plasmids
Lg-Cps-37R	TCTTGTCCTCCAGAGGGTTCTCCT	CPS plasmids
Lg-Cps-38R	ATGTTTCCCGTGTCCGTGCATC	CPS plasmids
Lg-Cps-38F	CCATTCCGACACGAAACTGATC	Chromosome (Lg2 type)
Lg-Cps-36R	ATCACTACGGTTGAGGATCATG	Chromosome (Lg2 type)
Lg-Cps-40F	GCTCCTGATGTAACCTATGCTTC	Chromosome (NUF462 type)
Lg-Cps-40R	CTGATGCTGCTGCATATGTTGAC	Chromosome (NUF462 type)

* The position of the primers was illustrated in Figs. 4, 5 and 6.

according to the manufacturer's instructions. The capsule synthetic gene locus of each strain was fragmentally amplified by PCR and sequenced as previously described (Kanai *et al.*, 2017). The upstream and downstream regions of capsule gene cluster of each strain were amplified using an inverse PCR technique and sequenced as previously described (Kanai *et al.*, 2017). The prediction of open reading frames and homology search on the newly found sequences were performed using DNASIS software (Hitachi software) and BLAST program at National Center for Biotechnology Information (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), respectively. ClustalW2.1 network version at DNA Data Bank of Japan (<http://clustalw.ddbj.nig.ac.jp/index.php?lang>) was used for DNA multiple sequence alignment.

The sequence data of the NUF462 chromosomal capsule gene cluster with franking region and CPS plasmids, pNUF18, pNUF462 and pNs15sousui29, are available on DDBJ/GenBank/EMBL databases under accession numbers LC324727, LC316979, LC316980 and LC316981, respectively.

Identification of CPS plasmids and integration site of chromosomal capsule gene cluster

The presence and differentiation of CPS plasmids in the *L. garvieae* strains were investigated by a PCR method using *TaKaRa Ex Taq* Polymerase (Takara Bio). Primer pairs used for amplification were Lg-pF2/Lg-Cps-37R and Lg-pF2/Lg-Cps-38R for the upper region of the capsule gene cluster and Lg-Cps-1F/Lg-pR2 for the lower region of the capsule gene cluster (Table 3). The

PCR reaction mixture (50 μ L) contained 5 μ L of 10 \times PCR buffer for *TaKaRa Ex Taq* Hot Start Version, 0.4 mM of each dNTP, 0.5 μ M of each primer, 1 μ L of template DNA and 1.25 U of *TaKaRa Ex Taq* Hot Start Version. The PCR condition was as follows; initial denaturation at 95°C for 3 min and 30 cycles of denaturation at 98°C for 10 s, annealing at 65°C for 30 s and extension at 72°C for 3 min. The expected sizes of PCR products using the above-mentioned primer pairs were 3,325 and 1,296 bp, 3,325 and 1,296 bp, 4,330 and 2,301 bp and 4,330 and 2,301 bp for the upper region and 1,541, 2,737, 1,541 and 526 bp for the lower region of pNUF18, pNUF462, pBSLG13015 and pNs15sousui29, respectively.

The integration site of the chromosomal capsule gene cluster in the strains was investigated by a long PCR using KOD FX Neo Polymerase mentioned above. Primer pairs used were Lg-Cps-38F/Lg-Cps-36R for the Lg2 type integration site and Lg-Cps-40F/Lg-Cps-40R for the NUF462 type integration site (Table 3).

Antimicrobial susceptibility test

Susceptibility of the *L. garvieae* strains to erythromycin and lincomycin was studied using microplate-cultivation method. Briefly, fresh culture of each strain was inoculated into 150 μ L of Muller-Hinton broth supplemented with erythromycin (25 μ g/mL) or lincomycin (5 μ g/mL) in a well of a 96-well microplate and incubated for 24 h at 27°C. Strains that grew in the wells were judged to be resistant to respective antibiotics. The bacterial growth in each well was assessed from turbidity measured at 600 nm with a microplate reader, Multi-

skan FC (Thermo Scientific).

Virulence test

The two colony types of *L. garvieae* NUF462, opaque and relatively transparent, were cultured on TH agar for 24 h. The bacterial suspensions were prepared in 0.01 M phosphate buffered saline, pH 7.2 (PBS), serially 10 fold-diluted with PBS and intramuscularly injected to ten yellowtail per dilution at a rate of 0.1 mL/100 g body weight. The control fish were injected with PBS. The average body weight of experimental fish was 57.6 g. After inoculation, ten fish received the same dilution were stocked in a 100-L circular aquarium with constant water flow and reared for 3 wk with feeding. The average water temperature was 25.7°C.

Results

Relationship between colony morphology and serological phenotype

When the stocked *L. garvieae* KG⁻ strains were cultured on TH agar, two colony types, namely, opaque colony and relatively transparent one, grew from many strains like filefish isolates (Fig. 2). The results of slide

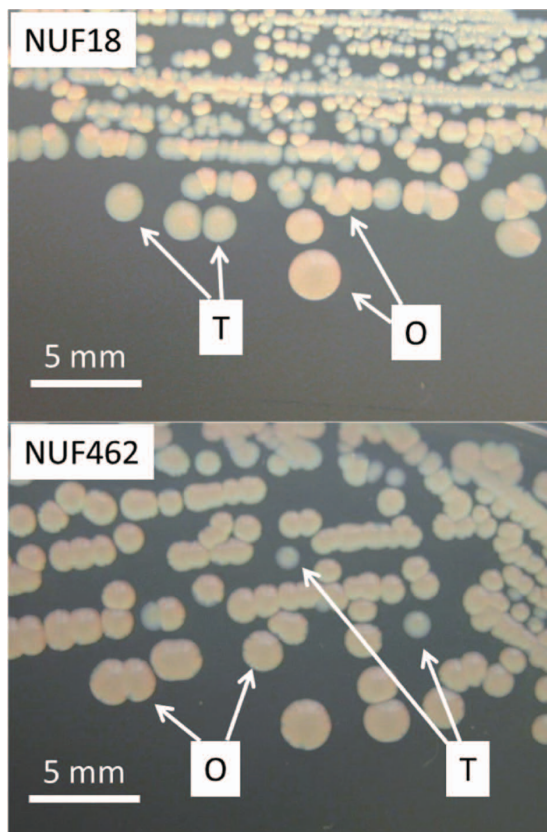


Fig. 2. Opaque (O) and relatively transparent (T) colonies of *L. garvieae* NUF18 (group 1) and NUF462 (group 2) grown on Todd Hewitt agar cultured for 2 days at 27°C. The photographs were taken with a transmitted light that obliquely illuminated the agar plates.

agglutination test revealed that the strains were divided into three groups. In one group opaque colonies were KG⁻ type, while relatively transparent colonies were KG⁺ type (group 1). In the second group both colony types were KG⁻ type (group 2). Strains that exhibited only one colony morphology also existed, and such strains were revealed to be KG⁻ type (group 3).

Detection of capsule gene cluster in the genome of *L. garvieae* strains

In the strains belonged to group 1, opaque colonies (KG⁻ type) exhibited the plasmid capsule gene cluster, while relatively transparent colonies (KG⁺ type) did not. The chromosomal capsule gene cluster was not detected in either colony type (Fig. 3). On the other hand, in several strains belonged to group 2, opaque colonies (KG⁻ type) exhibited the plasmid capsule gene cluster, but relatively transparent colonies (KG⁻ type) exhibited neither the plasmid nor chromosomal capsule gene cluster. Then the integration site of the capsule gene cluster in the relatively transparent colonies of a representative strain, *L. garvieae* NUF462, was investigated, and it was revealed that the gene cluster was integrated in the chromosome at a position different from *L. garvieae* Lg2. Moreover, it was found that in the NUF462 opaque colonies the chromosomal gene cluster was detected at the same position as the relatively transparent colonies, that is, the opaque colonies of NUF462 was revealed to possess both the plasmid and chromosomal capsule gene clusters simultaneously (Fig. 3). In the rest of group 2 strains both opaque and relatively transparent colonies exhibited only the chromosomal capsule gene cluster at the same position as Lg2. In the KG⁻ strains that exhibited only one colony morphology (group 3) the plasmid or chromosomal capsule gene cluster at the same position as NUF462 or Lg2 was detected.

The plasmid or chromosomal capsule gene cluster was detected in some KG⁺ strains. In such cases, it is possible that mutation occurred in the capsule synthetic genes, but we did not elucidate this in this study.

Structure of integration site of chromosomal capsule gene cluster

The chromosomal capsule gene cluster of NUF462 was flanked by two IS982 elements like Lg2 and integrated in the spacer region between LCGL_1603 and LCGL_1604 of the Lg2 genome (GenBank accession no. NC_017490). A DNA sequence consisted of nine nucleotides, ACAATAAAA, which seemed to be a target sequence existed at both sides of the inserted capsule gene cluster plus IS982 elements (Fig. 4). In Lg2 and the other representative strains, NUF18, Ns12sousui23 and Ns15sousui29, this short sequence existed singly. Similarly, in the case of integration site in Lg2, a sequence consisted of AATATACAA was found at both

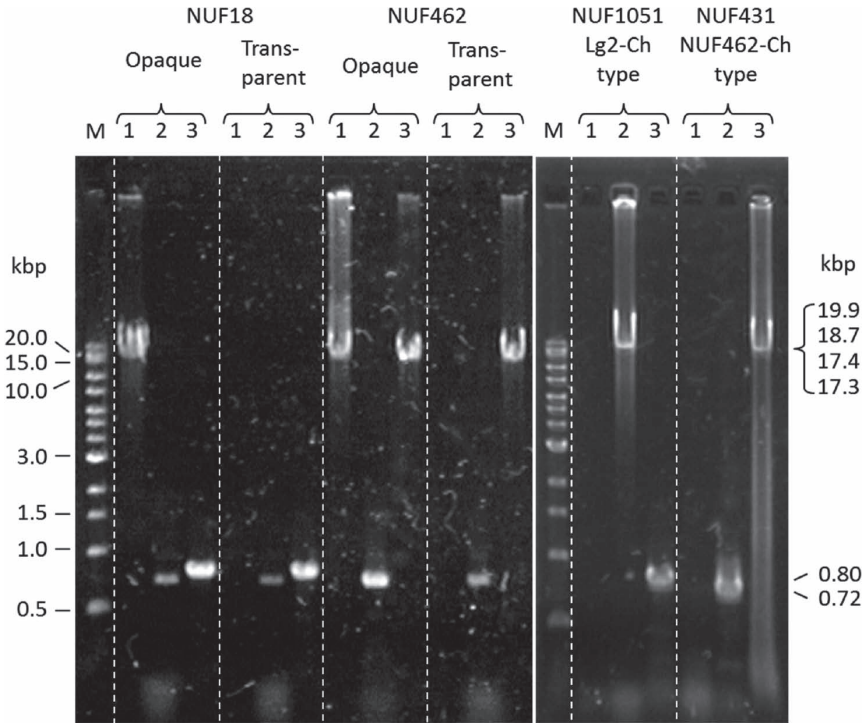


Fig. 3. Detection of plasmid and chromosomal capsule gene clusters in *L. garvieae* NUF18 opaque (KG^- , pNUF18 type) and relatively transparent colonies (KG^+ type), NUF462 opaque (KG^- , pNUF462 & NUF462-Ch type) and relatively transparent colonies (KG^- , NUF462-Ch type), NUF1051 (KG^- , Lg2-Ch type) and NUF431 (KG^- , NUF462-Ch type) by PCR. 1, plasmid capsule gene cluster. Appearance of a band at 18.7 and 19.9 kbp indicates the presence of the gene cluster (pNUF18 and pNUF462 type, respectively). 2, Lg2 type chromosomal capsule gene cluster (Lg2-Ch type). Appearance of bands at 17.3 kbp and 720 bp indicates the presence and absence of the gene cluster, respectively. 3, NUF462 type chromosomal capsule gene cluster (NUF462-Ch type). Appearance of bands at 17.4 kbp and 800 bp indicates the presence and absence of the gene cluster, respectively. M, DNA size marker.

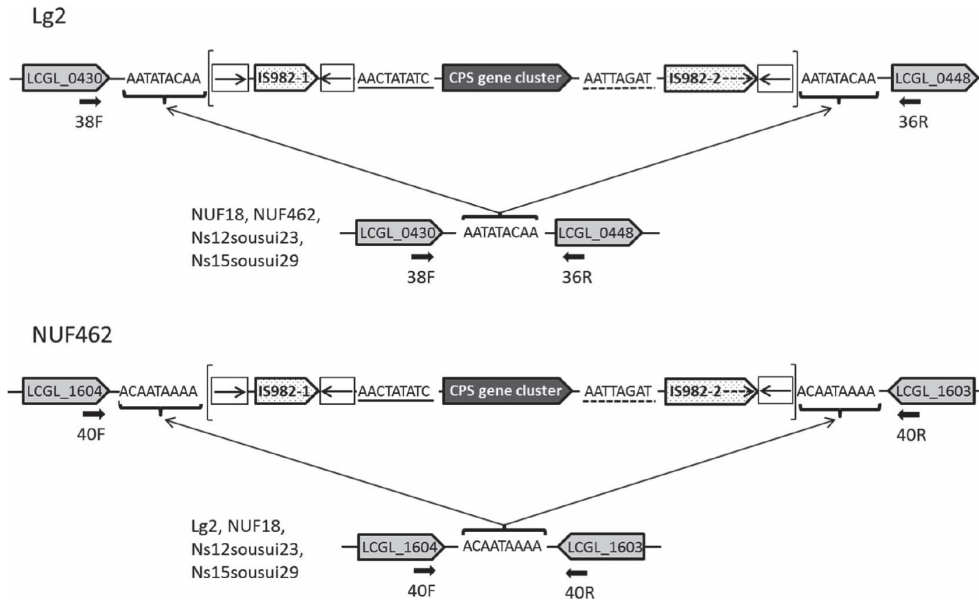


Fig. 4. Structure of the integration site of chromosomal capsule gene cluster in *L. garvieae* Lg2 and NUF462. Genes are not drawn to scale. The figure of Lg2 was drawn based on the sequence data of GenBank accession no. NC_017490. Sequence in brackets is a cassette structure also observed in CPS plasmids. Underlined sequences consisted of eight or nine nucleotides are target sequences detected in CPS plasmids (see Fig. 6). \rightarrow and \leftarrow , inverted repeat sequences, which are also detected in CPS plasmids, (see Fig. 6). Short arrows indicate the position and direction of PCR primers used for detection and differentiation of the integration site of chromosomal capsule gene cluster: 38F, Lg-Cps-38F; 36R, Lg-Cps-36R; 40F, Lg-Cps-40F; 40R, Lg-Cps-40R.

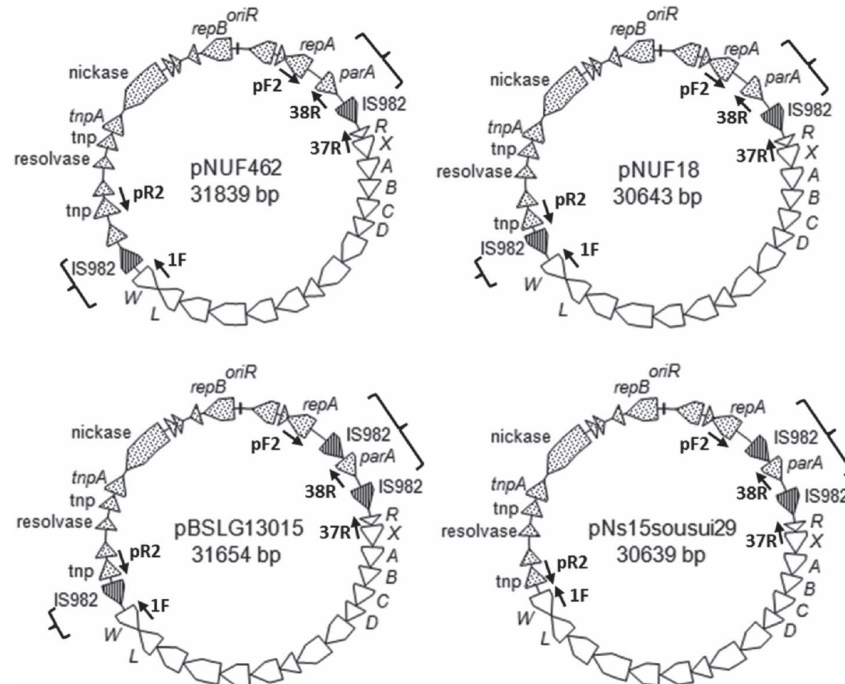


Fig. 5. Structure of the CPS plasmids pNUF462, pNUF18, pBSLG13015 (Kanai *et al.*, 2017) and pNs15sousui29. Boxes with no pattern are the genes of capsule gene cluster. *R* to *W*, *epsR*, *epsX*, *epsA*, *epsB*, *epsC*, *epsD*, *epsL* and *epsW*. *tnp*, transposase. Arrows indicate the position and direction of PCR primers used for detection and differentiation of CPS plasmids: pF2, Lg-pF2; pR2, Lg-pR2; 1F, Lg-Cps-1F; 37R, Lg-Cps-37R; 38R, Lg-Cps-38R. Curly braces indicate the variable regions.

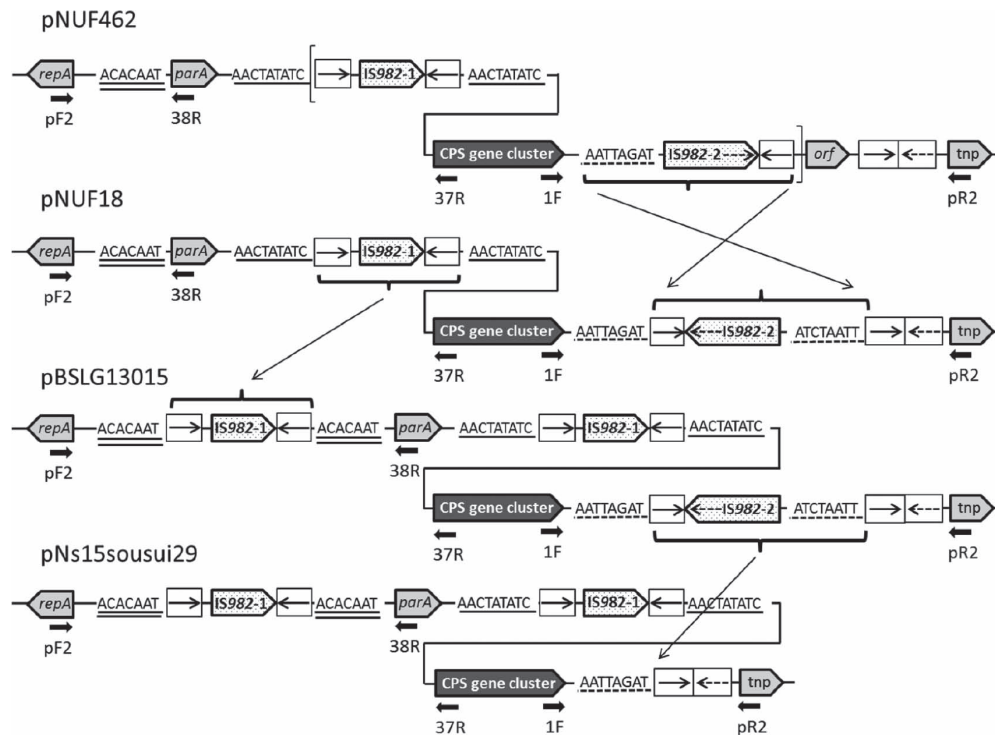


Fig. 6. Structure of upstream and downstream regions adjacent to the capsule gene cluster of CPS plasmids. Genes are not drawn to scale. Sequence in brackets is a cassette structure also observed in the chromosome. Underlined sequences consisted of seven to nine nucleotides are target sequences for IS982 element. \rightarrow and \leftarrow , inverted repeat sequences, ACCCGAATTGCTAGTTGATT and TTCTATCGGGTATCAAGTCATGGCTAAAT, respectively. Short arrows indicate the position and direction of PCR primers used for detection and differentiation of CPS plasmids: pF2, Lg-pF2; pR2, Lg-pR2; 1F, Lg-Cps-1F; 37R, Lg-Cps-37R; 38R, Lg-Cps-38R.

sides of the capsule gene cluster. Two kinds of inverted repeat sequences consisted of 20 and 29 nucleotides, respectively, were found adjacently to IS982 elements (Fig. 4).

Structure of CPS plasmids

Sequence analyses of plasmids from the four representative strains, NUF18, NUF462, Ns12sousui23 and Ns15sousui29, resulted in the discovery of three novel CPS plasmids from respective strains, designated pNUF18, pNUF462 and pNs15sousui29 (Fig. 5). The plasmid from Ns12sousui23 had the same structure as the CPS plasmid pBSLG13015 from filefish (Accession no. LC205736). The structure of these four plasmids resembled each other, but the upstream and downstream regions adjacent to the capsule gene cluster were different. The direction of two IS982 elements that sandwiched the capsule gene cluster in pNUF462 was the same as the chromosomal gene cluster of NUF462 and Lg2. In pNUF18 and pBSLG13015 the downstream IS982 was settled in the opposite direction compared to pNUF462. In pNs15sousui29 and pBSLG13015 another IS982 element was inserted between *repA* and *parA* genes. In Ns15sousui29 the downstream IS982 was lost. Like the integration sites of chromosomal capsule gene cluster, sequences consisted of seven to nine nucleotides were found to sandwich IS982 elements. The two kinds of repeat sequences consisted of 20 and 29 nucleotides were found to sandwich IS982 elements inversely or directly (Fig. 6).

Detection of CPS plasmids and integration site of chromosomal capsule gene cluster among the strains

The results of PCR for the detection and differentiation of the integration site of chromosomal capsule gene cluster and CPS plasmids are shown in Figs. 3 and 7. By the PCR the stocked *L. garvieae* strains were differentiated into eight categories (Fig. 8). Strains that possessed pNUF18 or pNUF462 (pNUF18 and pNUF462 type, respectively) were found till the year of 1991. Strains whose capsule gene cluster was integrated in the chromosome at the same position as NUF462 (NUF462-Ch type) were found between 1989 and 1992, most of which also possessed pNUF462 (pNUF462 & NUF462-Ch type). Strains whose capsule gene cluster was integrated in the chromosome at the same position

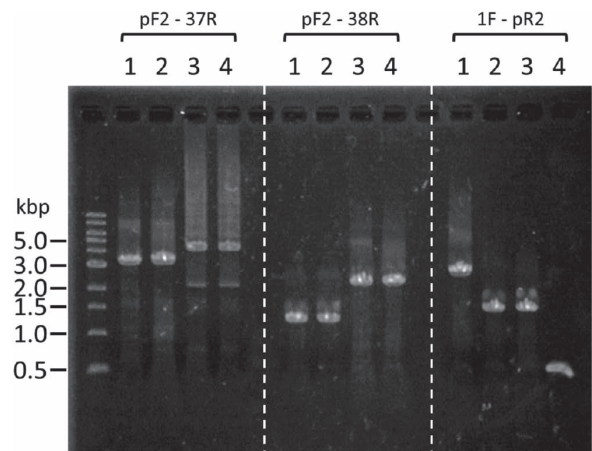


Fig. 7. Differentiation of CPS plasmids by PCR. 1, pNUF462; 2, pNUF18; 3, pBSLG13015; 4, pNs15sousui29. pF2, Lg-pF2; pR2, Lg-pR2; 1F, Lg-Cps-1F; 37R, Lg-Cps-37R; 38R, Lg-Cps-38R.

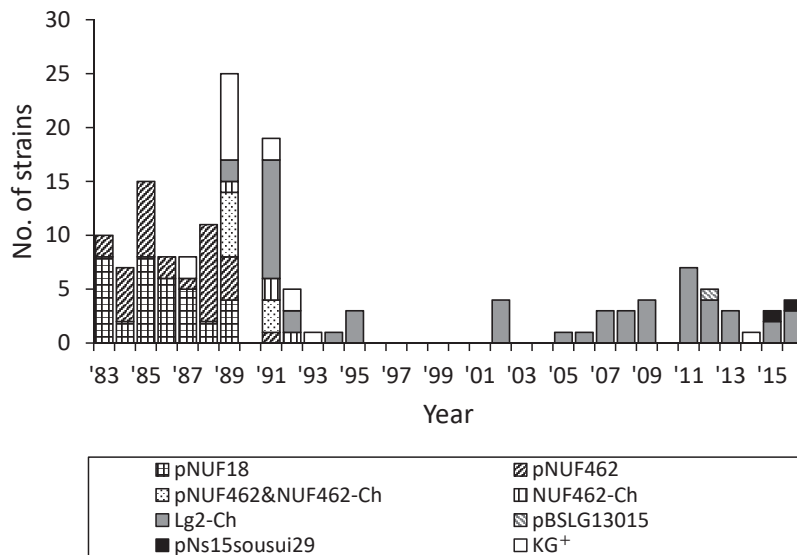


Fig. 8. Distribution of CPS plasmids (pNUF18, pNUF462, pBSLG13015, pNs15sousui29) and chromosomal capsule gene cluster (NUF462-Ch, Lg2-Ch) among *L. garvieae* strains. Neither CPS plasmids nor chromosomal capsule gene cluster were detected in the strains categorized as KG⁺.

Table 4. Antibiotic susceptibility of the *L. garvieae* strains

Integration site of capsule gene cluster	Total no. of strain	Susceptibility to:			
		Erythromycin		Lincomycin	
		S	R	S	R
pNUF18	35	35	0	35	0
pNUF462	31	30	1	30	1
pNUF462 & NUF462-Ch	9	2	7	2	7
NUF462-Ch	4	1	3	1	3
Lg2-Ch					
1989–1994	16	16	0	10	6
1995–2016	38	38	0	1	37
pBSLG13015	1	1	0	1	0
pNs15sousui29	2	2	0	2	0

S, susceptible; R, resistant; Ch, chromosome.

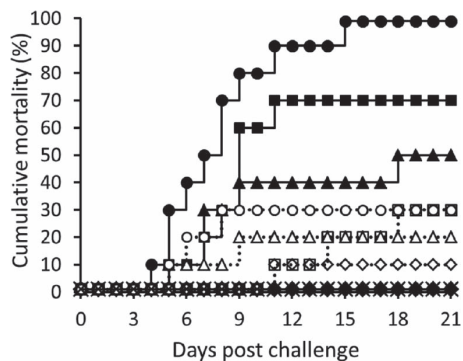


Fig. 9. Virulence of opaque (●, ▲, ■, ◆; 1.6×10^4 – 10^1 CFU/100 g body weight, respectively) and relatively transparent colonies (○, △, □, ◇; 1.6×10^4 – 10^1 CFU/100 g body weight, respectively) of *L. garvieae* NUF462 to yellowtail studied by intramuscular inoculation. The control fish (x) were injected with PBS.

as Lg2 (Lg2-Ch type) were found between 1989 and 2016. pBSLG13015 was detected only in Ns12sousui23 isolated from Pacific bluefin tuna, and pNs15sousui29 was detected in two strains isolated from kingfish *S. lalandi* in 2015 (Ns15sousui29) and 2016, but the other strains derived from these fish species belonged to Lg2-Ch type. Strains categorized as KG⁺, in which neither CPS plasmids nor chromosomal capsule gene cluster were detected, were supposed to be KG⁺ derivatives of CPS plasmid type (Fig. 8).

Relationship between integration site of capsule gene cluster and antibiotic susceptibility

As shown in Table 4, almost all the strains of pNUF18 and pNUF462 types were susceptible to erythromycin and lincomycin, but the majority of strains of NUF462-Ch and pNUF462 & NUF462-Ch types were resistant to both antibiotics. In Lg2-Ch type one-third of strains isolated between 1989 and 1994 were resistant to lincomycin, and almost all the strains isolated between 1995 and 2016 were resistant to this antibiotic. Strains possessed pBSLG13015 and pNs15sousui29 were susceptible to both antibiotics.

Virulence of opaque and relatively transparent colonies of NUF462 to yellowtail

The mortalities in the fish groups inoculated with the opaque colonies from 10^4 to 10^2 CFU/100 g body weight were higher than those inoculated with the relatively transparent colonies (Fig. 9). The result indicates that the opaque colonies of NUF462 are more virulent than the relatively transparent ones.

Discussion

In this study we have shown that the integration site of the capsule gene cluster of *L. garvieae* was varied among the strains tested. Some strains possessed one of the four CPS plasmids, and the others possessed the capsule gene cluster in the chromosome at the same position as *L. garvieae* NUF462 or Lg2. Moreover, some strains simultaneously possessed both the CPS plasmid and chromosomal capsule gene cluster. In *L. lactis* several kinds of gene cluster for exopolysaccharide production have been found, and their integration sites differ according to gene cluster, namely in plasmids or chromosome (Morita *et al.*, 2011). In *L. garvieae* the capsule gene cluster in the plasmids and chromosome was the same, suggesting that the structure and serological properties of the capsular polysaccharide produced are the same despite that the integration sites are different. Previously we reported that all the *L. garvieae* strains isolated from filefish possessed the CPS plasmid pBSLG13015 and thought that the CPS plasmid was restricted to filefish isolates (Kanai *et al.*, 2017). However, from the present study it is certain that CPS plasmids are widely distributed among the Japanese *L. garvieae* isolates not only from filefish but from the other fish species.

There seems to be a transition of the integration site of the capsule gene cluster. In this study pNUF18 and pNUF462 were detected in the strains isolated between 1983 and 1991. Their serological phenotypic change from KG⁻ to KG⁺ type occurred by subculturing probably due to plasmid curing. Since the phenotypic change

from KG^- to KG^+ is a well-known character of *L. garvieae* (Kitao, 1982), the majority of *L. garvieae* isolated before 1983 are also supposed to be the plasmid type. The strains of NUF462-Ch and Lg2-Ch types appeared in 1989, and the Lg2-Ch type prevailed till 2016. Their serological phenotypic change seemed to occur at a relatively low frequency compared to the strains possessing CPS plasmids (Fig. 1). A previous paper reported the frameshift mutations existed in the capsule synthetic genes of *L. garvieae* Lg2-S, a KG^+ derivative of Lg2 (Morita *et al.*, 2011). In the present study not a few KG^+ strains belonged to Lg2-Ch type were found (data not shown). So, in cases of the *L. garvieae* isolates possessing the chromosomal capsule gene cluster the phenotypic change may occur due to mutation in the capsule synthetic genes rather than loss of the gene cluster.

The four *L. garvieae* CPS plasmids described in this paper had similar structure, but the upstream and downstream regions adjacent to the capsule gene cluster were varied among them. The comparison of the variable regions indicate that the transition of structure in the order of pNUF462 \rightarrow pNUF18 \rightarrow pBSLG13015 \rightarrow pNs-15sousui29 might have occurred by inversion, replication and deletion of the IS982 element. It is possible that the chromosomal capsule gene cluster may be translocated from pNUF462, because 1) the structure of a cassette consisted of capsule gene cluster plus two IS982 elements in the chromosome is the same as that of pNUF462, 2) the earlier *L. garvieae* strains tended to possess the CPS plasmids only, 3) both pNUF462 and the chromosomal capsule gene cluster co-existed in some strains, and 4) the repeat sequence consisted of 20 nucleotides was situated inversely at the both ends of the cassette structure like as IS982 element (Fig. 4). It is known that the inverted repeat sequences of ISs act as a receptor for transposase, so they are essential for the transposition of IS elements (Szabó *et al.*, 2010).

It is interesting that the susceptibility of the *L. garvieae* strains to erythromycin and lincomycin was related to the integration site of the capsule gene cluster. Multiple drug resistant *L. garvieae* including macrolide resistance emerged and prevailed at yellowtail aquaculture sites around 1990, and, recently, lincomycin-resistant *L. garvieae* isolates have increased (Aoki *et al.*, 1990; Nishiki *et al.*, 2011; Furushita *et al.*, 2015). It is possible that the integration site might have influenced the acquisition of resistance, or, conversely, the acquisition of resistance might have affected the change in the integration site. To solve this question a further study will be needed by increasing the number of *L. garvieae* isolates examined.

In this study the two colony morphologies, opaque and relatively transparent, were observed among the *L. garvieae* strains as filefish isolates (Kanai *et al.*, 2017). These colony morphologies probably represent encapsulated or non-encapsulated, respectively, in the strains

possessing the CPS plasmid only such as NUF18 and filefish isolates. In this case the colony opacity may reflect encapsulation. On the other hand, in the strains such as NUF462, although the two colony types were observed, both the types were KG^- phenotype, namely encapsulated. It is possible that the colonies possessing both the CPS plasmid and chromosomal capsule gene cluster may produce capsular polysaccharide more abundantly and become opaquer than the colonies possessing the chromosomal gene cluster only. The abundance of capsular polysaccharide may be related to the virulence. In the present study opaque colonies of NUF462 were revealed to be more virulent than transparent ones. If the copy number of CPS plasmids in a bacterial cell is more than two, the amount of capsular polysaccharide produced may increase. In a previous study we have shown that the virulence of a *L. garvieae* strain isolated from filefish which possesses the CPS plasmid pBSLG13015 was higher than that isolated from yellowtail, whose capsule gene cluster is integrated in the chromosome only (Kanai *et al.*, 2017). In conclusion, it is suggested that *L. garvieae* isolates possessing CPS plasmids form opaque colonies and are highly virulent compared to those possessing the chromosomal capsule gene cluster only.

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